Investigation of the Active Site of Aminopeptidase A Using a Series of New Thiol-Containing Inhibitors

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Aminopeptidase A (APA) and aminopeptidase N (APN) are two metallopeptidases which have been suggested to be involved in the enzymatic cascade of the renin–angiotensin system. APA liberates angotensin III from angiotensin II by releasing the N-terminal aspartate, and APN participates in the inactivation of angiotensin III. As the role of angiotensin III in the regulation of blood pressure in the central nervous system and at the periphery is controversial, it was of interest to develop selective and efficient inhibitors of APA. Starting from Glu-thiol,[1] which was the first efficient APA inhibitor described, but however is equipotent on APA (K_i = 0.14 μM) and APN (K_i = 0.12 μM), β-amino thiols bearing various carboxyalkyl chains have been synthesized and their inhibitory potencies measured on both purified enzymes. Compounds containing a carboxylated aromatic ring inhibited APA and APN with K_i values in the micromolar range but were slightly more active on APA. Conversely, inhibitors containing a cyclohexyl ring were more efficient on APN. Various modifications of the structure of Glu-thiol decreased inhibitory activity on both enzymes but increased the selectivity for APA, and compound **9d** ((*S*)-4-amino-6-mercaptohexanoic acid) was 23 times more potent on APA (K_i = 2.0 μM) than on APN (K_i = 45 μM).

Introduction

Aminopeptidase A (APA, EC 3.4.11.7), discovered by Glenner in 1961,[2] is an integral membrane type II ectopeptidase, which specifically cleaves the peptide bond following N-terminal Glu and Asp residues.[3] APA hydrolyses α-L-Glu-β-naphthylamide (GluNA) at 5 times the rate of α-L-Asp-β-naphthylamide and is activated by alkaline-earth metals (Ca^{2+}, Ba^{2+}, Sr^{2+}).[4,5] The murine surface antigen BP-1/6C3, which is a marker of differentiation of pre-B cells in the bone marrow, was recently cloned by Wu et al.[6] and shown to correspond to APA.[7] Another group has also recently cloned human APA from renal carcinoma cells in culture.[8] From these studies, it appears that APA is a member of the zinc-dependent metallopeptidase superfamily[9,10] that contains a consensus sequence H-E-X-X-H in which the two histidines coordinate the zinc atom and the glutamate is involved in catalysis (for a review, see ref 11). Aminopeptidase N (APN, EC 3.4.11.2) is another member of this family. Not surprisingly, APA and APN share a certain sequence homology (33%)[6,8] and are often colocalized, which led to difficulties in early attempts to purify APA.[12] In contrast to APA, APN has a wide specificity, directed against N-terminal neutral and basic amino acids.[13] Both of these two enzymes seem to be involved in the metabolism of angiotensins.[14] APA has been suggested to be responsible for the transformation of angiotensin II (AII) to des-Asp^1-AII (angiotensin III, AIII),[15–17] and APN has been proposed to inactivate AIII by cleaving its N-terminal Arg.[18,19] APA, when administered iv, lowers blood pressure,[20] but icv administration has the opposite effect, which is greatly diminished by sarthan, a specific angiotensin antagonist.[18] This suggests that AIII may be the true effector of AII receptors in the central nervous system (CNS) (for a review of the central renin–angiotensin system, see ref 21) where APA has also been evidenced.[22,23] As these data are still controversial, specific inhibitors of APA would be interesting pharmacological tools to study the brain angiotensin system. Only a few inhibitors of APA have been described in the literature. The first one, amastatin,[24] described as a specific inhibitor of APA (IC_{50} = 8 μM), is in fact 40 times more potent on APN (IC_{50} = 0.2 μM).[19] Using the approach developed for the synthesis of aminopeptidase inhibitors,[25–27] Wilk and Thurston[1] have recently described two compounds. Glu-thiol and Asp-thiol, which inhibit APA with K_i values of 0.4 and 1.2 μM, respectively. Given the high specificity of APA for its natural substrates, these results are somewhat surprising. Indeed, as compared to Lys-thiol for arginylaminopeptidase (K_i = 0.9 nM)[26] or Leu-thiol (K_i = 51 nM)[25] or Met-thiol (K_i = 11 nM)[27] for aminopeptidase N, Glu-thiol and Asp-thiol are relatively poor inhibitors of APA. Furthermore, as underlined by Wilk and Thurston, these two compounds inhibit APN with about the same affinity (K_i = 0.25 and 7.5 μM, respectively) in spite of the hydrophobic character of the S_1 subsite of APN.[14]

With the aim of synthesizing potent and selective inhibitors of APA, we have followed the strategy used for the rational design of metallopeptidase inhibitors.[28] As a first step, we have explored its S_1 subsite with various β-amino thiols containing carboxylated side chains. Their inhibitory potencies were determined for both APA and APN in order to establish the parameters which would clearly differentiate between these two enzymes.

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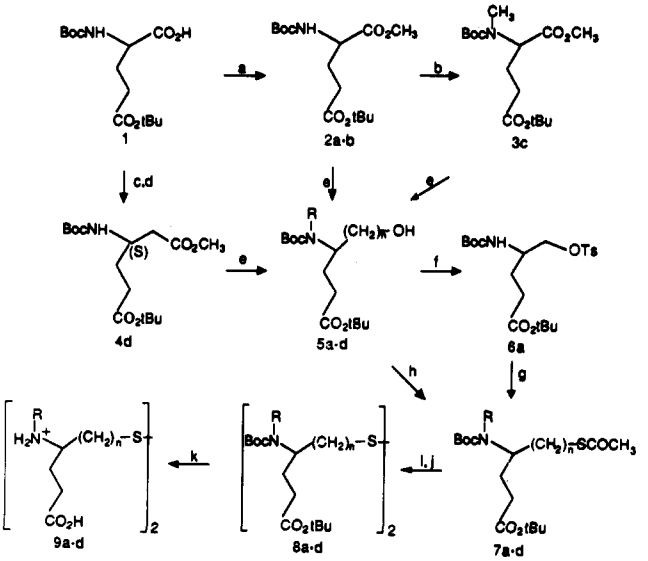


Figure 1. Scheme for the synthesis of the aliphatic inhibitors: (a) Cs_2CO_3, CH_3I; (b) CH_3I, NaH; (c) iBuOCOCl, CH_2N_2, *N*-ethylmorpholine; (d) $PhCO_2Ag$, MeOH; (e) $NaBH_4$; (f) TsCl; (g) CH_3COSK; (h) PPh_3, $(iPrN{=})_2$, CH_3COSH; (i) NaOH; (j) I_2; (k) TFA.

Results

Chemistry. The synthesis of the glutamate derivatives is summarized in Figure 1. From the commercially available BocGlu(OtBu)OH, three types of compounds were synthesized, the two stereoisomers of Glu-thiol **9a** and **9b**, the *N*-methyl analogue **9c**, and the corresponding γ-amino thiol **9d**.

For the synthesis of (*S*)- and (*R*)-Glu-thiol, the α-carboxylate of **1** was selectively reduced in alcohol **5a** or **5b** via the methyl esters **2a** and **2b**. The thioacetylation was performed either by activation of the alcohol, as tosylate, followed by substitution with potassium thioacetate or by the Mitsunobu reaction.[29] The fully protected compounds, **7a** and **7b**, led to the inhibitors **9a** and **9b** in two steps: saponification of the thioester group and isolation of the disulfide **8a** and **8b**, followed by acidic cleavage of the *tert*-butyl ester and Boc groups by TFA.

The same synthetic pathway was used for the *N*-methyl derivative **9c** and the γ-amino thiol **9d**. The N-methylation was performed at the beginning of the synthesis by treatment of **2a** with CH_3I in the presence of NaH, and the homologization of **1a** was classically performed using the Arndt–Eistert method giving the *N*-Boc and amino diester **4d** with retention of configuration.

Given that there were two ester groups present, the critical step of the synthesis was the reduction of the α-ester function to alcohol. As found for the aliphatic compounds (Figure 1), the protection of the side-chain carboxylate by a *tert*-butyl allowed the reduction to be completely selective for the α-ester. For the other β-amino thiols described (compounds **28a–h**), the *N*-Boc-amino diesters are not commercially available and had to be synthesized (see Figures 2 and 3). However, the protection of the side-chain carboxylate by a *tert*-butyl gave a very low yield in these series. The protection by a methyl or an ethyl was almost quantitative but raised the problem of the selectivity of the reduction of the α-ester. The carboxylic ester was therefore reduced using sodium borohydride, a method which although not generally applicable,[30,31] can be used when activating substituents are present,[32] as is the case with the *N*-Boc-amino esters. The α-ester is thus

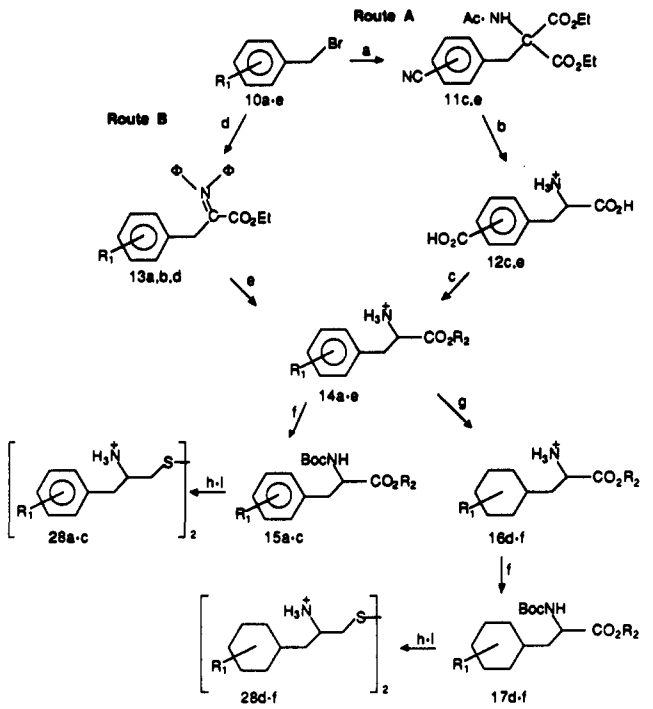


Figure 2. Scheme for the synthesis of the phenylalanine derivatives: (a) $AcNHCH(CO_2Et)_2$, EtONa; (b) 6 N HCl, Δ; (c) $SOCl_2$, MeOH; (d) $Ph_2C{=}NCH_2CO_2Et$, $PhCH_2N(CH_3)_3OH$; (e) 1 N HCl, Et_2O; (f) $(Boc)_2O$; (g) H_2, PtO_2, P, Δ; (h) $NaBH_4$; (i) PPh_3, $(iPrN{=})_2$, CH_3COSH; (j) NaOH; (k) I_2; (l) TFA.

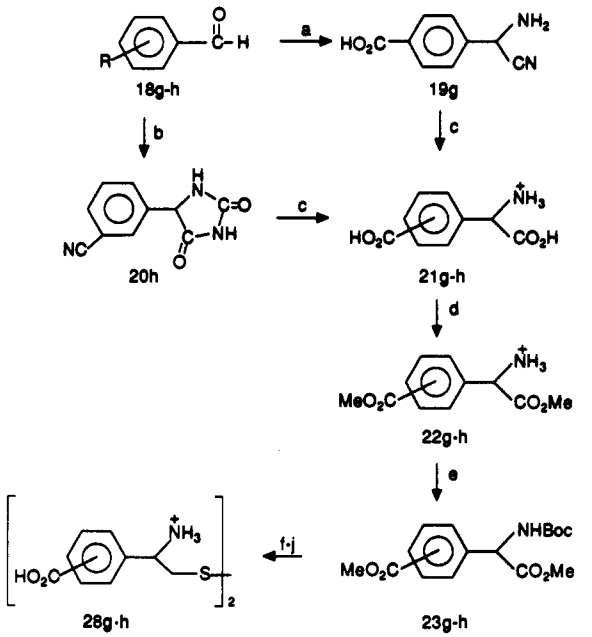
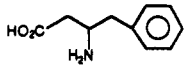
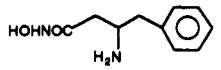
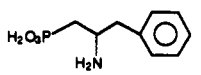
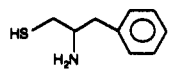


Figure 3. Scheme for the synthesis of the phenylglycine derivatives: (a) NaCN, NH_4Cl; (b) $(NH_4)_2CO_3$, KCN; (c) 6 N HCl, Δ; (d) $SOCl_2$, MeOH; (e) $(Boc)_2O$; (f) $NaBH_4$; (g) PPh_3, $(iPrN{=})_2$, CH_3COSH; (h) NaOH; (i) I_2; (j) TFA.

reduced before the lateral one in all cases. The reaction was stopped before completion, and the three compounds, the starting diester, the monoalcohol and dialcohol, produced were well separated by TLC. An easy purification could thus be performed and the monoalcohol was identified by NMR (δ ppm 3.3–3.5 CH_2OH, 4.55–4.65 OH), as compared to the dialcohol (δ ppm, 3.10–3.20 side-chain CH_2OH, 4.20–4.30 side-chain OH).

The synthesis of the substituted cyclic derivatives of alanine is described in Figure 2. The starting material

Table 1. Comparison of the Inhibition of APA and APN by Phenylalanine Derivatives

	formula	% inhibn APA at 0.4 mM	APN[a] IC_{50} (μM)
A		0	800 ± 100
B		0	75 ± 5
C		13	500 ± 80
D		85	0.030 ± 0.004

[a] From ref 27.

was variously substituted benzyl bromides. Two main routes were used. Route A consisted of alkylation with diethyl acetamidomalonate, followed by hydrolysis with 6 N HCl and esterification with thionyl chloride in ethanol. Route B started with a phase-transfer alkylation using the diphenylimine of glycine. After treatment with 1 N HCl in ether, the amino diesters obtained were protected on the α-amine with a Boc group. Alternatively, the aromatic amino diesters were subjected to hydrogenation to yield the cyclohexyl derivatives, which were then amino-protected.

Figure 3 shows the synthesis of the substituted cyclic analogues of glycine. Two methods of amino acid construction were used starting from substituted benzaldehydes. Strecker and Bucherer–Bergs syntheses,[33] followed by hydrolysis with 6 N HCl gave the desired α-amino acid functions. All these cyclic amino acids were transformed into β-amino thiols by the synthetic pathway described in Figure 1.

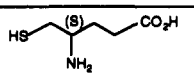
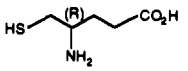
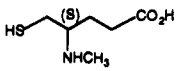
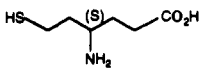
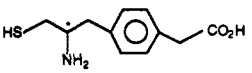
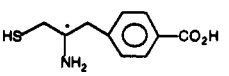
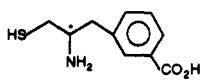
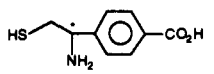
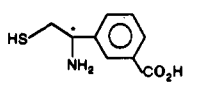
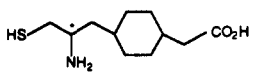
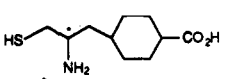
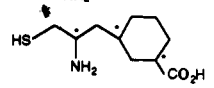
Inhibitory Potencies. Previously described[27] phenylalanine derivatives, bearing various functional groups able to interact with the zinc atom in the catalytic domain of APA, were tested (Table 1). β-Phenylalanine **A** and β-phenylalanine hydroxamate **B** did not inhibit APA at concentrations of 0.4 mM. However, the phosphonate and the thiol analogs **C**, **D** were more active, giving 13% and 85% inhibition, respectively, of APA at the same concentration. The K_i value calculated for Phe-thiol was 39 μM. The sulfhydryl group thus appeared to be the best Zn ligand to obtain a strong inhibition of the enzyme.

The K_i values of the different compounds synthesized are reported in Table 2. For APA, (*S*)-Glu-thiol was the most efficient inhibitor, with a K_i value of 0.14 μM. In the aliphatic series, all the modifications performed, i.e., change in the stereochemistry, methylation of the α-amino group, and displacement of the sulfhydryl group in position γ, led to K_i values ranging from 0.87 to 2.3 μM.

Inhibitors containing aromatic or cyclohexyl side chains also had similar micromolar activity on APA, whatever the position of the carboxylate group on the cyclic moiety, except for **28g** and **28f**, which were in the 10 μM range. The inhibition of APN by the aliphatic derivatives showed that Glu-thiol was also the most efficient in this series, but a greater modulation of activity was observed as a function of the modification introduced. The (*R*) absolute configuration in **9b** and the thiol displacement in **9d** gave the most dramatic changes in APN inhibition.

In the benzylic series **28a–c** the position of the carboxylate did not induce large changes in APN inhibition.

Table 2. Inhibitory Potencies of Various Thiol Inhibitors for APA and APN

		K_i (μM)	
no.	formula	APA	APN
9a		0.14 ± 0.06	0.12 ± 0.02
9b		2.3 ± 0.7	17 ± 3
9c		0.87 ± 0.4	4.5 ± 0.6
9d		2.0 ± 1.2	45 ± 5
28a		2.2 ± 1.1	2.7 ± 0.5
28b		2.7 ± 0.2	7.3 ± 0.7
28c		0.84 ± 0.2	3.85 ± 0.7
28g		>3	25 ± 6
28h		2.4 ± 1.6	1.9 ± 0.6
28d		2.8 ± 0.7	0.035 ± 0.002
28e		1.9 ± 0.4	2.3 ± 0.3
28f		>3	0.21 ± 0.07

However, it was interesting to observe that a carboxylate group in the para position was three times less active that a carboxymethyl group in the same position. This effect was more sensitive in the phenylglycine series **28g,h**, in which the presence of a carboxylate in the para position led to a significant loss of activity (K_i = 25 μM).

The greatest differences were obtained in the cyclohexyl series: again the presence of a carboxylate directly on the cycle in **28e** led to a K_i value in the micromolar range, whereas the presence of a carboxymethyl group led to the best inhibitory potency on APN for this series (compound **28d**, K_i = 35 nM).

Discussion

In order to explore the active site of APA and to compare its specificity with that of APN, various β-amino thiols were synthesized. The sulfhydryl group was chosen as the zinc ligand from the results reported in Table 1. This preference for an SH group seems to be a common feature for Zn aminopeptidases, since the best inhibitors of aminopeptidase N and aminopeptidase B also contain this metal coordinating group.[25-27] However, the phosphonate **C** has an inhibitory potency on APA of the same order of magnitude as the thiol **D**, unlike APN which is poorly recognized by this compound.

Conversely, the hydroxamate **B** has a better activity on APN than on APA. This seems to reflect an important characteristic of the active site of APA and is another

interesting difference from APN. Two types of hydroxamate bearing compounds have been tested on APN: C-terminal hydroxamates such as compound **B** or "N-terminal" hydroxamates such as kelatorphan, $HONHCOCH_2CH(CH_2C_6H_4)CONHCH(CH_3)COOH$ and analogs,[34,35] which interact with the S'_1 and S'_2, subsites of the enzyme. Inhibitory potencies as high as 10 nM have been obtained for such compounds, with a K_i value of 0.38 μM for kelatorphan. For APA, the two types of hydroxamate compounds are poor inhibitors, with no inhibition at 0.4 mM for **B** and a K_i value of 0.16 mM for kelatorphan.

From the inhibitory potencies of compounds **9a–d** for APA and APN reported in Table 2, other differences in the two catalytic sites could be deduced. As shown by the K_i values of **9b–d** for APN, this peptidase has, as previously reported,[27] a constrained active site. As compared to (*S*)-Glu-thiol (**9a**), large decreases in activity were obtained by inversion of the absolute configuration in **9b** (factor 100), by N-methylation of the free amino group in **9c** (factor 40), or by the displacement of the thiol group in **9d** (factor 400). Conversely for APA, the same modifications are less stringent, since 16- and 14-fold decreased potencies were measured for **9b** and **9d**, respectively, and only a 6-fold decrease for **9c**. Thus, **9d** is a relatively selective inhibitor, being 23 times more potent on APA than on APN.

Furthermore, the data reported in Table 2 give various indications of the differences between the S_1 subsites of APA and APN. As previously reported by Wilk,[1] Glu-thiol is a relatively potent APA inhibitor, but it is equipotent on APN (0.12 μM). This is not completely unexpected since compounds containing an aliphatic chain, such as Leu-thiol or Met-thiol, inhibit APN in the 10 nM range.[25,27] Consequently, the presence of the hydrophilic carboxylate decreases the recognition of APN, but only by a factor of 10. The same conclusions can be drawn from the results with the cyclic inhibitors: indeed Phe-thiol, phenylglycinethiol, and cyclohexylalaninethiol inhibit APN in the 25–45 nM range,[27] and the introduction of a carboxylate on the cyclic moities in compounds **28a–h** significantly decreases APN inhibition, with only one exception: compound **28d**, which was a relatively good inhibitor of APN with a K_i of 35 nM. This suggests an interaction between the well positioned COOH group of **28d** and a positively charged group or acceptor present in the S_1 subsite of APN. Moreover, these molecules, in spite of the presence of a carboxylate group, have relatively hydrophobic side chains which are probably not very well accepted by the S_1 subsite of APA. However, as compared to Phe-thiol (K_i = 39 μM), the presence of the carboxylate in **28h** did not induce any change, but it increased the recognition of the APA active site at least 10-fold in **28a–c,h**. Consequently, it is possible that the addition of other hydrophilic groups on the cycle of this aromatic series might increase both the affinity and the selectivity for APA.

Furthermore, even though this aromatic series was not very efficient, it shows the interest of exploring the possibility of a geometrically well defined interaction between the carboxylate and a positively charged group of the S_1 subsite of APA. The slight increase in APA inhibition for **28c** (0.84 μM) as compared to **28a,b,g,h** seems to indicate that the meta position is a relatively favorable orientation and suggests that the introduction of a second carboxylate group in this position could reinforce APA recognition as well as selectivity. This result seems to be at variance with the distance observed between the α-carbon and the carboxylate in Glu-thiol, which would correspond to an ortho-substituted aromatic ring. The synthesis of other derivatives in the aliphatic or aromatic series will be useful to define more precisely the optimized distance between these two groups.

In conclusion, the present study is a preliminary step in the characterization of the active site of APA, as compared to APN. From the results obtained, it would seem that this peptidase has a relatively large active site at the level of both the catalytic domain and the S_1 subsite. These data were unexpected as, due to the high specificity of the enzyme for substrates bearing a N-terminal aspartate or glutamate residue, it could have been assumed that only a restricted number of structures would be accepted by its active site. As compared to Glu-thiol, which is at the present the most efficient APA inhibitor, compound **28c**, although containing a bulkier chain, is a relatively efficient inhibitor with a K_i of 0.4 μM for the most active stereoisomer. This result is interesting as, if the accumulation of carboxylates allows an increase in both efficacy and selectivity, this type of aromatic molecule can easily be labeled by a radioactive atom (^3H or ^{125}I), possibly leading to a probe for binding and autoradiography experiments. On the other hand, compound **9d** is a selective inhibitor of APA with a K_i value (2.0 μM), 23 times lower thant its K_i value for APN (45 μM). Taking the results of this study into account, introduction of the structural and functional parameters leading to affinity and selectivity for APA inhibition in a new series of compounds is in progress.

Experimental Section

Inhibitory Potency. Aminopeptidase A, purified from rabbit kidney as described by Wilk and Thurston,[1] hydrolyzed α-L-glutamyl-β-naphthylamide (GluNA) at a rate of 100 μmol mL^{-1} h^{-1}. GluNA (K_m = 130 μM for APA) was from Bachem. Aminopeptidase N from hog kidney was purchased from Boehringer Mannheim (Meylan, France) and was suspended in 3.2 M ammonium sulfate, 50 mM Tris buffer, pH 7.4. [^{3}H]Tyr^1-Leu^5-enkephalin (30 Ci/mmol, K_m = 50 μM for APN) was from Amersham. The solutions of the thiol inhibitors were prepared in Tris buffer pH 7.4 containing DTT (100 equiv/equiv of inhibitor). The K_i values were calculated from IC_{50} values using the Cheng–Prusoff relationship.[36]

Aminopeptidase A. We used the procedure of Goldbarg[37] with a downscale modification on a microplate. APA was incubated for 1 h at 37 °C with or without increasing concentrations of inhibitors and with 200 μM GluNA, in a total volume of 100 μL in 50 mM Tris-HCl buffer pH 7.4 with 4 mM $CaCl_2$. The reaction was stopped by adding 10 μL of 3 N HCl. In order to determine by diazotization the 2-naphthylamine liberated by substrate hydrolysis, 25 μL of 87 mM (0.6%) $NaNO_2$ were then added, and after 3 min, 50 μL of 0.13 M (1.5%) ammonium sulfamate. After a further 5 min, 25 μL of a 23 mM (0.6%) solution of *N*-(1-naphthyl)ethylenediamine dihydrochloride in 95% EtOH was added, and the plate was incubated for 30 min at 37 °C. A standard curve was prepared in parallel by diazotizing increasing concentrations (up to 0.2 mM) of 2-naphthylamine in 0.1 N HCl. The absorbance was measured at 560 nm.

Aminopeptidase N. APN was preincubated for 15 min at 25 °C, with or without increasing concentrations of inhibitors, in a total volume of 100 μL in 50 mM Tris-HCl buffer pH 7.4 [^{3}H]-Tyr^1-Leu^5-enkephalin was added to a final concentration of 10 nM, and the reaction was stopped after 15 min by adding 10 μL of 0.5 M HCl. The tritiated metabolite [^{3}H]Tyr was separated on polystyrene beads, and the radioactivity was measured by liquid scintillation counting, as described by Vogel and Altstein.[38]

Chemistry. Amino acids were obtained from Bachem (Bubendorf, Switzerland). All the other reagents were obtained from Aldrich (Saint Quentin Fallavier, France). The solvents were from Merck (Nogent sur Marne, France).

Melting points of the crystallized compounds were taken on an electrothermal apparatus and are reported uncorrected. Chromatography was carried out with Merck silica gel (230–400 mesh). TLC was performed on precoated silica gel plates (60F-254, 0.2 mm thick, Merck) with the following solvent systems (*v/v*): (A) CH_2Cl_2–MeOH, 9:1; (B) *n*-hexane–EtOAc, 6:4; (C) CH_2Cl_2–MeOH–AcOH, 9:1:0.5; (D) *n*-hexane–EtOAc–AcOH, 5:5:0.5; (E) $CHCl_3$–MeOH–H_2O–AcOH, 5:5:1:0.5. Plates were developed with UV light, iodine vapor, or ninhydrin. The purity of the final compounds was also checked by HPLC using a Spherisorb silica column 5 μm, 80 Å (Touzart & Matignon, Vitry sur Seine, France) with CH_2Cl_2–MeOH–AcOH as solvent. The eluted peaks were monitored at 236 nm. The structure of the compounds was confirmed by ^1H NMR spectroscopy on a Bruker AC (270 MHz) in DMSO-d_6 using HMDS as internal reference, and satisfactory analyses (<±0.4%) were obtained (C, H, N) for all compounds.

The following abbreviations are used: MeOH, methanol; EtOH, ethanol; EtOAc, ethyl acetate; THF, tetrahydrofuran; Boc, *tert*-butyloxycarbonyl; Boc_2O, di-*tert*-butyl dicarbonate; Et_2O, diethyl ether; DMF, *N,N*-dimethylformamide; Ts, tosyl. Other abbreviations used are those recommended by the IUPAC–IUB commission (*Biochem. J.* 1984, *219*, 345).

General Procedure for Protection of the Amino Group. The amino group was protected with a *tert*-butyloxycarbonyl group with the classical method in DMF,[39] procedure A.

General Procedures for Protection of the Carboxylate Group. The *tert*-butyl esters were prepared using *N,N*-dimethylformamide di-*tert*-butyl acetal as described by Widmer,[40] procedure B.1. The methyl or ethyl esters were prepared in acidic conditions by the Fischer method (alcohol + $SOCl_2$), procedure B.2, or in basic conditions via the cesium salt of the acid and CH_3I,[41] procedure B.3.

General Procedures for Reduction of the Ester Function: Procedure C.1. The Boc-amino diester was dissolved in EtOH–water (1:1) (3 mL/mmol) and $NaBH_4$ (1 equiv) in the same solvent was added dropwise at 0 °C.[32] After 15 min, the mixture was heated at 50 °C and stirred for 2–4 h. Then the EtOH was evaporated, and the resulting solution was extracted with EtOAc, washed with brine, dried over Na_2SO_4, and evaporated to dryness.

Procedure C.2. The Boc-amino diester (1 equiv) was dissolved in dry EtOH–THF (8 mL/mmol) and cooled to 0 °C. $NaBH_4$ (4 equiv) and LiCl (4 equiv) in solution in the same solvent were added dropwise at 0 °C.[42] The mixture was then allowed to warm to room temperature and stirred overnight. The reaction was stopped with 1 N HCl, extracted with EtOAc, washed with water, 1 N HCl, $NaHCO_3$, and brine, dried over Na_2SO_4, and evaporated in vacuo.

General Procedures for Substitution of the Hydroxyl Group: Procedure D.1. Triphenylphosphine (2 equiv) was dissolved in dry THF (3.5 mL/mmol). Diisopropyl azodicarboxylate (2 equiv) was added at 0 °C and the mixture stirred for 30 min until a light yellow precipitate formed. The alcohol (1 equiv) dissolved in THF (3 mL/mmol) and CH_3COSH (2 equiv) were then added. The temperature was allowed to rise slowly to room temperature while the mixture was stirred overnight. After evaporation in vacuo, the residue was dissolved in EtOAc and washed successively with a 10% $NaHCO_3$ solution, H_2O, and brine before being dried over Na_2SO_4. After evaporation, the residue was taken up in *n*-hexane–EtOAc, and the precipitate was eliminated. The filtrate was evaporated and the residue purified by flash chromatography on silica gel column, using *n*-hexane–EtOAc, 4:1, as eluent.

Procedure D.2. The alcohol (1 equiv) was dissolved in pyridine/KOH (1 mL/mmol), and 1.2 equiv of freshly recrystallized TsCl in pyridine/KOH (0.5 mL/mmol) was added at 0 °C. The mixture was stirred overnight at 4 °C. The precipitate was filtered off and the solution evaporated to dryness. The residue was taken up in EtOAc, washed with H_2O, citric acid, H_2O, and brine, dried over Na_2SO_4, and evaporated in vacuo. To a solution of the tosylate (1 equiv) in DMF (3 mL/mmol) was added at 0 °C 3 equiv of CH_3COSK (prepared from CH_3COSH and KOH in DMF). The mixture was stirred overnight at room temperature and evaporated to dryness. The residue was taken up in EtOAc, washed with water and brine, dried over Na_2SO_4, and evaporated in vacuo.

General Procedures for Deprotection Reactions. Procedure E.1: Saponification of Esters and Thioesters. The product (1 equiv) was dissolved in its corresponding alcohol (EtOH or MeOH) (5 mL/mmol), and 1N NaOH (2.5 equiv per ester to hydrolyze) was added at 0 °C. The mixture was stirred for 30 min at 0 °C and for 3 h at room temperature. A solution of I_2 in EtOH was added until a persistant yellow color was obtained. The excess of iodine was reduced by $Na_2S_2O_3$, and the solution was evaporated in vacuo. The residue was taken up in water, acidified with 3 N HCl, and extracted with EtOAc. The organic layer was washed with $Na_2S_2O_3$, H_2O, and brine, dried over Na_2SO_4, and evaporated to dryness.

Procedure E.2: Deprotection of Boc and *tert*-Butyl Esters Groups by TFA. The product (1 equiv) was dissolved in CH_2Cl_2 (3 mL/mmol). At 0 °C were added anisole (1 equiv per *tert*-butyl group) and TFA (10 equiv per *tert*-butyl group). The mixture was stirred for 30 min at 0 °C and for 2 h at room temperature. Cyclohexane was added to facilitate the evaporation of the TFA in vacuo. The residue was taken up with cyclohexane and evaporated to dryness three or four times.

Procedure E.3: General Deprotection by Refluxing 6 N HCl. The product was taken up in a large excess of 6 N HCl, and the mixture was heated at 130 °C and stirred overnight. Then the mixture was allowed to cool down, before being evaporated to dryness. The residue was dissolved in H_2O and evaporated again. This process was repeated three or four times to eliminate excess acid, before lyophilization.

Procedure E.4: Deprotection of the Diphenylimine. The compound (1 equiv) was dissolved in Et_2O (3 mL/mmol) and 1 N HCl (3 mL/mmol) and stirred for 4 h at room temperature.[43] The ethereal phase was then separated, and the aqueous layer was made alkaline with 10% $NaHCO_3$ and extracted seven times with CH_2Cl_2. The organic extracts were dried over Na_2SO_4 and evaporated in vacuo.

General Procedures for Amino Acid Synthesis: Procedure F.1. To a solution of Na (1.1 equiv) in EtOH (2.5 mL/mmol of Na) was added at 0 °C diethyl acetamidomalonate (1 equiv) and after 20 min the halogeno derivative (1 equiv). The solution was stirred for 30 min at room temperature and then warmed at 45 °C on an oil bath. After overnight stirring, the solution was concentrated to dryness and taken up with EtOAc. It was then washed with water and brine, dried over Na_2SO_4, and evaporated in vacuo. Whenever possible, the product was recrystallized in H_2O/EtOH.

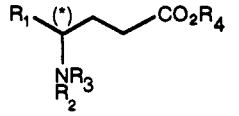
Procedure F.2. To a cold (10 °C) stirring solution of the halogeno derivative (1 equiv), ethyl *N*-(diphenylmethylene)-glycinate (1.1 equiv), and potassium iodide (0.11 equiv) in dioxane (9 mL/mmol) was added dropwise benzyltrimethylammonium hydroxide (40% solution in water) (0.4 mL/mmol).[44] The reaction mixture was then brought to room temperature and stirred for 3 h. After cooling at 0 °C, water (8 mL/mmol) was added and the mixture was extracted six times with toluene. The organic extracts were washed with water, dried over $CaCl_2$, and evaporated in vacuo.

Procedure F.3 (Strecker Reaction). A solution of NaCN (1 equiv) and NH_4Cl (1.1 equiv) was stirred in water while the aldehyde (1 equiv) in MeOH was added. The mixture was refluxed for 1 h and then stirred overnight at room temperature before evaporation to dryness.

Procedure F.4 (Bucherer–Bergs Reaction). The substituted benzaldehyde was dissolved in MeOH–H_2O, 1:1 (3 mL/mmol). $(NH_4)_2CO_3$ (5 equiv) was added, followed by KCN (1.1 equiv). The mixture was stirred for 18 h at 55 °C. It was evaporated to dryness after cooling and then taken up in H_2O + EtOAc. After filtration, the organic phase was separated and washed by brine and evaporated to dryness.

General Procedure for Hydrogenation. The aromatic compound was dissolved in 80% AcOH (5 mL/mmol), and PtO_2 (100 mg/mmol) was added in a hydrogenation autoclave. The hydrogen pressure was set to 80 bars and the temperature to 80 °C. The reaction was shaken overnight and then evaporated in

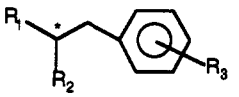
Table 3. Physical Constants of Aliphatic Compounds[a]



no.	R_1	R_2	R_3	R_4	*	method	yield (%)	mp (°C)	TLC
2a	CO_2Me	Boc	H	tBu	*S*	B.3	95	oil	0.62 (B)
5a	CH_2OH	Boc	H	tBu	*S*	C.2	93	oil	0.20 (B)
7a	CH_2SAc	Boc	H	tBu	*S*	D.2	51	oil	0.60 (B)
8a	$CH_2S-]_2$	Boc	H	tBu	*S*	E.1	93	oil	0.59 (B)
9a	$CH_2S-]_2$	TFA, H	H	H	*S*	E.2	92	84	0.19 (E)
2b	CO_2Me	Boc	H	tBu	*R*	B.3	96	65	0.63 (B)
5b	CH_2OH	Boc	H	tBu	*R*	C.2	95	oil	0.23 (B)
7b	CH_2SAc	Boc	H	tBu	*R*	D.1	59	oil	0.60 (B)
8b	$CH_2S-]_2$	Boc	H	tBu	*R*	E.1	92	oil	0.61 (B)
9b	$CH_2S-]_2$	TFA, H	H	H	*R*	E.2	67	85	0.20 (E)
3c	CO_2Me	Boc	Me	tBu	*S*	*b*	15	oil	0.56 (B)
5c	CH_2OH	Boc	Me	tBu	*S*	C.2	88	oil	0.44 (A)
7c	CH_2SAc	Boc	Me	tBu	*S*	D.1	50	oil	0.69 (B)
8c	$CH_2S-]_2$	Boc	Me	tBu	*S*	E.1	44	oil	0.63 (B)
9c	$CH_2S-]_2$	TFA, H	Me	H	*S*	E.2	70	>260	0.56 (E)
4d	CH_2CO_2Me	Boc	H	tBu	*S*	c	62	oil	0.64 (B)
5d	CH_2CH_2OH	Boc	H	tBu	*S*	C.2	94	oil	0.24 (B)
7d	CH_2CH_2SAc	Boc	H	tBu	*S*	D.1	79	oil	0.65 (B)
8d	$CH_2CH_2S-]_2$	Boc	H	tBu	*S*	E.1	60	oil	0.60 (B)
9d	$CH_2CH_2S-]_2$	TFA, H	H	H	*S*	E.2	72	81	0.21 (E)

[a] See the Experimental Section for methods and TLC systems. * = (*R*,*S*). [b] From ref 45. [c] From ref 42.

Table 4. Physical Constants of Phenylalanine Derivatives[a]



no.	R_1	R_2	R_3	method	yield (%)	mp (°C)	TLC
13a	CO_2Et	$N{=}CPh_2$	*p*-CH_2CO_2tBu	F.2	53	oil	0.70 (B)
14a	CO_2Et	NH_2	*p*-CH_2CO_2tBu	E.4	72	oil	0.51 (A)
15a	CO_2Et	NHBoc	*p*-CH_2CO_2tBu	A	97	oil	0.64 (B)
24a	CH_2OH	NHBoc	*p*-CH_2CO_2tBu	C.1	50	oil	0.48 (A)
26a	CH_2SAc	NHBoc	*p*-CH_2CO_2tBu	D.2	13	oil	0.62 (B)
27a	$CH_2S-]_2$	NHBoc	*p*-CH_2CO_2tBu	E.1	71	oil	0.60 (B)
28a	$CH_2S-]_2$	NH_2, TFA	*p*-CH_2CO_2H	E.2	89	201	0.29 (E)
13b	CO_2Et	$N{=}CPh_2$	*p*-CO_2tBu	F.2	92	oil	0.68 (B)
14b	CO_2Et	NH_2	*p*-CO_2tBu	E.4	50	oil	0.40 (A)
15b	CO_2Et	NHBoc	*p*-CO_2tBu	A	92	oil	0.66 (B)
24b	CH_2OH	NHBoc	*p*-CO_2tBu	C.1	42	121	0.24 (B)
26b	CH_2SAc	NHBoc	*p*-CO_2tBu	D.1	66	oil	0.63 (B)
27b	$CH_2S-]_2$	NHBoc	*p*-CO_2tBu	E.1	72	60	0.71 (B)
28b	$CH_2S-]_2$	NH_2, AcOH	*p*-CO_2H	E.2	70	165	0.25 (E)
11c	$(CO_2Et)_2$	NHAc	*m*-CN	F.1	65	139	0.22 (B)
12c	CO_2H	NH_2, HCl	*m*-CO_2H	E.3	100	>260	0.32 (E)
14c	CO_2Me	NH_2, HCl	*m*-CO_2Me	B.2	90	oil	0.71 (E)
15c	CO_2Me	NHBoc	*m*-CO_2Me	A	79	oil	0.74 (A)
24c	CH_2OH	NHBoc	*m*-CO_2Me	C.1	48	oil	0.51 (A)
26c	CH_2SAc	NHBoc	*m*-CO_2Me	D.1	56	oil	0.63 (B)
27c	$CH_2S-]_2$	NHBoc	*m*-CO_2H	E.1	68	209	0.56 (D)
28c	$CH_2S-]_2$	NH_2, TFA	*m*-CO_2H	E.2	68	76	0.24 (E)
13d	CO_2Et	$N{=}CPh_2$	*p*-CH_2CO_2Et	F.2	79	78	0.71 (B)
14d	CO_2Et	NH_2	*p*-CH_2CO_2Et	E.4	83	oil	0.45 (A)
11e	$(CO_2Et)_2$	NHAc	*p*-CN	F.1	57	164	0.62 (A)
12e	CO_2H	NH_2, HCl	*p*-CO_2H	E.3	90	>260	0.50 (E)

[a] See the Experimental Section for methods and TLC systems.

vacuo. The residue was taken up in cyclohexane and evaporated again a few times to eliminate AcOH (procedure G).

The physical constants and the procedures used to obtain the different compounds are listed in Tables 3–6. Additional data concerning the end-products follow.

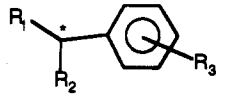
(*S*)-5,5′-Dithiobis(4-aminopentanoic acid), Bis(trifluoroacetate) (9a). Anal. ($C_{10}H_{20}N_2O_4S_2$·2TFA), C, H, N. HPLC (CH_2Cl_2–MeOH–AcOH, 7:3:0.1): t_R 4.7 min. ^{1}H NMR (DMSO + TFA): δ 1.74–1.99 (m, 2 H, CH_2CH), 2.38 (t, 2 H, CH_2CO), 2.8–3.06 (m, 2 H, CH_2S), 3.3–3.47 (m, 1 H, CHα), 7.92 (bs, 3 H, NH_3^+).

(*R*)-5,5′-Dithiobis(4-aminopentanoic acid), *Bis*(trifluoroacetate) (9b). Anal. ($C_{10}H_{20}N_2O_4S_2$·2TFA) C, H, N. HPLC (CH_2Cl_2–MeOH–AcOH, 7:3:0.1): t_R 4.7 min. ^{1}H NMR (DMSO + TFA): δ 1.86 (m, 2 H, CH_2CH), 2.38 (t, 2 H, CH_2CO), 2.88 and 3.02 (dd, 2 H, CH_2S), 3.4 (m, 1 H, CH), 7.97 (s, 3 H, NH_3^+).

L-5,5′-Dithiobis[4-(*N*-methylamino)pentanoic acid], Bis(trifluoroacetate) (9c). Anal. ($C_{12}H_{24}N_2O_4S_2$·2TFA), C, H, N. HPLC (CH_2Cl_2–MeOH–AcOH, 8:2:0.1) t_R 5.2 min. ^{1}H NMR (DMSO + TFA): δ 1.9 (m, 2 H, CH_2CH), 2.4 (t, 2 H, CH_2CO), 2.55 (m, 3 H, CH_3N), 3.1 (dd, 2 H, CH_2S), 3.4 (m, 1 H, CH), 8.0–8.5 (m, 2 H, NH_2^+).

(*S*)-6,6′Dithiobis(4-aminohexanoic acid), Bis(trifluoroacetate) (9d). Anal. ($C_{12}H_{24}N_2O_4S_2$·2TFA), C, H, N. HPLC (CH_2Cl_2–MeOH–AcOH, 8:2:0.1): t_R 5.8 min. ^{1}H NMR (DMSO + TFA): δ 1.65–1.78 (m, 2 H, CH_2CH_2CO), 1.76–1.9 (m, 2 H,

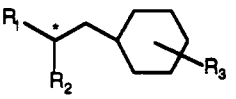
Table 5. Physical Constants of Phenylglycine Derivatives[a]



no.	R_1	R_2	R_3	method	yield (%)	mp (°C)	TLC
19g	CN	NH_2	*p*-CO_2H	F.3	52	>260	0.51 (C)
21g	CO_2H	NH_2, HCl	*p*-CO_2H	E.3	82	>260	0.55 (A)
22g	CO_2Me	NH_2, HCl	*p*-CO_2Me	B.2	49	>260	0.75 (E)
23g	CO_2Me	NHBoc	*p*-CO_2Me	A	45	oil	0.56 (B)
24g	CH_2OH	NHBoc	*p*-CO_2Me	C.1	30	oil	0.49 (A)
26g	CH_2SAc	NHBoc	*p*-CO_2Me	D.1	45	98	0.57 (B)
27g	$CH_2S-]_2$	NHBoc	*p*-CO_2H	E.1	50	oil	0.47 (D)
28g	$CH_2S-]_2$	NH_2, TFA	*p*-CO_2H	E.2	72	>260	0.46 (E)
20h	–CONHCONH–		*m*-CN	F.4	71	55	0.55 (A)
21h	CO_2H	NH_2, HCl	*m*-CO_2H	E.3	73	oil	0.39 (E)
22h	CO_2Me	NH_2, HCl	*m*-CO_2Me	B.2	85	oil	0.55 (A)
23h	CO_2Me	NHBoc	*m*-CO_2Me	A	78	oil	0.55 (B)
24h	CH_2OH	NHBoc	*m*-CO_2Me	C.2	86	oil	0.55 (A)
26h	CH_2SAc	NHBoc	*m*-CO_2Me	D.1	66	81	0.61 (B)
28h	CH_2SH	NH_2, HCl	*m*-CO_2H	E.3	94	124	0.62 (E)

[a] See the Experimental Section for methods and TLC systems.

Table 6. Physical Constants of Alicyclic Derivatives[a]



no.	R_1	R_2	R_3	method	yield (%)	mp (°C)	TLC
16d	CO_2Et	NH_2	4-CH_2CO_2Et	G	100	oil	0.45 (A)
17d	CO_2Et	NHBoc	4-CH_2CO_2Et	A	96	oil	0.77 (A)
24d	CH_2OH	NHBoc	4-CH_2CO_2Et	C.1	39	oil	0.38 (A)
26d	CH_2SAc	NHBoc	4-CH_2CO_2Et	D.1	67	oil	0.68 (B)
27d	$CH_2S-]_2$	NHBoc	4-CH_2CO_2H	E.1	89	86	0.52 (D)
28d	$CH_2S-]_2$	NH_2, TFA	4-CH_2CO_2H	E.2	69	128	0.50 (E)
14e	CO_2H	NH_2, HCl	4-CO_2H	G	100	oil	0.39 (A)
16e	CO_2Et	NH_2, HCl	4-CO_2Et	B.2	98	oil	0.37 (A)
17e	CO_2Et	NHBoc	4-CO_2Et	A	83	oil	0.65 (B)
24e	CH_2OH	NHBoc	4-CO_2Et	C.1	28	oil	0.41 (A)
26e	CH_2SAc	NHBoc	4-CO_2Et	D.1	58	oil	0.67 (B)
27e	$CH_2S-]_2$	NHBoc	4-CO_2H	E.1	71	60	0.54 (D)
28e	$CH_2S-]_2$	NH_2, TFA	4-CO_2H	E.2	70	87	0.45 (E)
14f	CO_2H	NH_2, AcOH	3-CO_2H	G	100	oil	0.44 (E)
16f	CO_2Me	NH_2, HCl	3-CO_2Me	B.2	98	oil	0.35 (A)
17f	CO_2Me	NHBoc	3-CO_2Me	A	89	oil	0.66 (B)
24f	CH_2OH	NHBoc	3-CO_2Me	C.1	25	oil	0.36 (A)
26f	CH_2SAc	NHBoc	3-CO_2Me	D.1	74	oil	0.69 (B)
27f	$CH_2S-]_2$	NHBoc	3-CO_2H	E.1	97	oil	0.53 (D)
28f	$CH_2S-]_2$	NH_2, TFA	3-CO_2H	E.2	68	85	0.48 (E)

[a] See the Experimental Section for methods and TLC systems.

CH_2CH_2S), 2.32 (t, 2 H, CH_2CO), 2.65–2.78 (m, 2 H, CH_2S), 3.12–3.23 (m, 1 H, CH), 7.77 (s, 3 H, NH_3^+).

(*R,S*)-1,1′-Dithiobis[2-amino-3-[*p*-(carboxymethyl)phenyl]propane], Bis(trifluoroacetate) (28a). Anal. ($C_{22}H_{28}N_2O_4S_2 \cdot 2TFA$) C, H, N. HPLC ($CH_2Cl_2$–MeOH–AcOH, 8:2:0.1): t_R 5.9 min. ^1H NMR (DMSO + TFA): δ 2.64–2.93 (m, 4 H, CH_2S + PhCH_2CH), 3.48 (s, 2 H, CH_2CO), 3.58 (m, 1 H, CH), 7.06–7.25 (m, 4 H, aromatic protons), 7.98 (s, 3 H, NH_3^+).

(*R,S*)-1,1′-Dithiobis[2-amino-3-(*p*-carboxyphenyl)propane], Diacetate (28b). Anal. ($C_{20}H_{24}N_2O_4S_2 \cdot 2AcOH$) C, H, N. HPLC ($CH_2Cl_2$–MeOH–AcOH, 8:2:0.1: t_R 6.4 min. ^1H NMR (DMSO + TFA): δ 1.83 (s, 3 H, $CH_3CO_2^-$), 2.65–3.04 (m, 4 H, CH_2S + PhCH_2CH), 3.64 (m, 1 H, CH), 7.3 and 7.88 (2 d, 2 × 2 H, aromatic protons), 8.03 (s, 3 H, NH_3^+).

(*R,S*)-1,1′-Dithiobis[2-amino-3-(*m*-carboxyphenyl)propane], Bis(trifluoroacetate) (28c). Anal. ($C_{20}H_{24}N_2O_4S_2 \cdot 2TFA$) C, H, N. HPLC ($CH_2Cl_2$–MeOH–AcOH, 8:2:0.1): t_R 5.8 min. ^1H NMR (DMSO): δ 2.8–3.0 (m, 4 H, CH_2S + $PhCH_2$), 3.65 (m, 1 H, CH), 7.45–7.8 (m, 4 H, aromatic protons), 8.1 (s, 3 H, NH_3^+).

(*R,S*)-1,1′-Dithiobis[2-amino-3-[4-(carboxymethyl)cyclohexyl]propane], Bis(trifluoroacetate) (28d). Anal. ($C_{22}H_{40}N_2O_4S_2 \cdot 2TFA$) C, H, N. HPLC ($CH_2Cl_2$–MeOH–AcOH, 8:2:0.1): t_R 5.6 min. ^1H NMR (DMSO + TFA): δ 0.87 (m, 1 H, C$H$$CH_2CH\alpha$), 1.15–1.74 (m, 10 H, $CHCH_2CH$ + 4 × CH_2 cyclohexyl), 1.8 (m, 1 H, C$H$$CH_2CO$), 2.01 and 2.11 (2 d, 2 H, CH_2CO), 2.91 (m, 2 H, CH_2S), 3.35 (m, 1 H, CH), 7.86 (s, 3 H, NH_3^+).

(*R,S*)-1,1′-Dithiobis[2-amino-3-(4-carboxycyclohexyl)propane], Bis(trifluoroacetate) (28e). Anal. ($C_{20}H_{36}N_2O_4S_2 \cdot 2TFA$) C, H, N. HPLC ($CH_2Cl_2$–MeOH–AcOH, 8:2:0.1): t_R 5.5 min. ^1H NMR (DMSO + TFA): δ 0.85 (m, 1 H, C$H$$CH_2CH\alpha$), 1.2–1.66 (m, 8 H, 4 × CH_2 cyclohexyl), 1.82 (m, 2 H, $CHCH_2CH$), 2.08 (m, 1 H, C$H$$CO_2H$), 2.4 and 2.95 (dd, 2 H, CH_2S), 3.35 (m, 1 H, $CH\alpha$), 7.85 (s, 3 H, NH_3^+).

(*R,S*)-1,1′-Dithiobis[2-amino-3-(3-carboxycyclohexyl)propane], Bis(trifluoroacetate) (28f). Anal. ($C_{20}H_{36}N_2O_4S_2 \cdot 2TFA$), C, H, N. HPLC ($CH_2Cl_2$–MeOH–AcOH, 8:2:0.1): t_R 5.4 min. ^1H NMR (DMSO + TFA): δ 0.7–1.0 (m, 1 H, C$H$$CH_2CH\alpha$), 1.1–2.0 (m, 8 H, 4 × CH_2, cyclohexyl), 2.1 (m, 3 H, C$H$$CO_2H$+$CHCH_2CH$), 2.9 (m, 2 H, CH_2S), 3.45 (m, 1 H, CH), 7.85 (s, 3 H, NH_3^+).

(*R,S*)-1,1′Dithiobis[2-amino-2-(*p*-carboxyphenyl)ethane], Bis(trifluoroacetate) (28g). Anal. ($C_{18}H_{20}N_2O_4S_2 \cdot 2TFA$) C, H, N. HPLC ($CH_2Cl_2$–MeOH–AcOH, 8:2:0.1): t_R 5.1 min. ^1H NMR (DMSO): δ 2.8–3.0 (m, 2 H, CH_2S), 3.65 (m, 1 H, CH), 7.45–7.8 (m, 4 H, aromatic protons), 8.1 (s, 3 H, NH_3^+).

(*R,S*)-2-Amino-1-mercapto-2-(*m*-carboxyphenyl)ethane, Hydrochloride (28h). Anal. ($C_9H_{11}NO_2S \cdot HCl$) C, H,

N. HPLC (CH_2Cl_2–MeOH–AcOH, 8:2:0.1): t_R 5.3 min. ^1H NMR (DMSO): δ 2.65 (t, 1 H, SH), 2.95 and 3.01 (m, 2 H, CH_2S), 4.4 (m, 1 H, CHα), 7.51 (t, 1 H, aromatic proton), 7.78 (d, 1 H, aromatic proton), 7.9 (d, 1 H, aromatic proton), 8.05 (s, 1 H, aromatic proton), 8.8 (bs, 3 H, NH_3^+).

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